Two Different Intrachain cAMP Binding Sites of cAMP-dependent Protein Kinases*

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The regulatory subunits of both isoforms of cAMP-dependent protein kinase bind 2 mol of cAMP/mol of monomer. cAMP dissociation studies indicate similar cAMP binding behavior for each isoform. Each has two different intrachain cAMP binding components present in approximately equal amounts and the rate of cAMP dissociation is 5- to 10-fold slower from one site (Site 1) than from the other (Site 2). Equilibrium [3H]cAMP binding is inhibited by several competing cyclic nucleotides. Following equilibrium binding using saturating [3H]cAMP in the presence of competing nucleotides, the pattern of release of [3H]cAMP, monitored in the presence of an excess of nonradioactive cAMP, suggests site-specific selectivity of some of the cyclic nucleotides. As compared with cAMP, cGMP prefers Site 2 for both regulatory subunits, whereas N\textsubscript{s}, O\textsubscript{6}-dibutyryl-cAMP shows a similar preference only with isoform II regulatory subunit. 8-Bromo-cAMP, 8-bromo-cGMP, and 8-azido-cAMP prefer Site 1 of both proteins. The results indicate that for each isoform the two intrachain binding sites have different analogue specificities and cAMP dissociation rates. Site 1 or Site 2 of one isoform has a similar but not identical cyclic nucleotide specificity and cAMP dissociation rate to the corresponding site of the other isoform.

A major intracellular receptor for cAMP in mammalian tissues is cAMP-dependent protein kinase (1). Both isoforms of this enzyme contain a regulatory (R) subunit dimer and two catalytic (C) subunits. R\textsuperscript{1} functions to inhibit C subunit activity, but this inhibition is relieved when cAMP binds to the R dimer, as follows (2): R\textsubscript{1}C\textsubscript{4} + 4 cAMP \rightleftharpoons R\textsubscript{4} (4 CAMP) + 2C. Work from this laboratory (2) first demonstrated the presence of two intrachain cAMP binding sites for the R subunit of cAMP-dependent protein kinase. This observation has recently been confirmed and extended to both isoforms I and II by Weber and Hliz (3) and by Smith et al. (4). Previously, nearly all studies directed toward understanding the mechanism of cAMP binding to the R subunits have been carried out under the assumption that the cyclic nucleotide binding stoichiometry was only 1 mol of cAMP/mol of monomeric R. Such studies have been thus subject to errors in interpretation. For example, positive cooperativity and the absence of cooperativity in cAMP binding to R have been reported (5, 6); however, the concept of intrachain rather than monomer-monomer binding site interaction has not been considered. Numerous cyclic nucleotide binding specificity studies have also been reported (7, 8). The radioactive photolinkage analogue, [3\textsuperscript{2}P]8-azido-cAMP, has been used to label the cAMP binding sites of the regulatory subunit (9, 10), although this derivative could have different affinities for the two intrachain sites. In fact, we show in this communication that for each isoform these intrachain sites differ in their cAMP dissociation rates as well as in their specificities for various cyclic nucleotides.

EXPERIMENTAL PROCEDURES

Purification of R Subunits—Isozyme II R subunit (R\textsubscript{II}) was prepared from fresh bovine hearts exactly as described before (2). Isozyme I R subunit (R\textsubscript{I}) was prepared from frozen rabbit skeletal muscle (Pel-Freez) by the same procedure used for R\textsubscript{II} with slight modifications. R\textsubscript{I} was eluted from DEAE-cellulose using 10 mm potassium phosphate (pH 6.8) and 1 mm EDTA (phosphate/EDTA) containing 0.15 m NaCl. The affinity gel used was 8(6-aminoethylamino)-cAMP-Sepharose 4B instead of 8(6-aminoethylamino)-cAMP-Sepharose 4B. The R\textsubscript{II} subunits used contained approximately 2 mol of bound cAMP/R subunit after purification and dialysis. All R subunit preparations were >95% pure as determined on 10% polyacrylamide disc gels containing SDS according to Weber and Osborn (11). Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard. Millipore filtration binding assays were performed as described before (2).

Materials—The 8(6-aminoethylamino)- and the 8(6-aminoethylamino)-cAMP-Sepharose 4B were purchased from P.L. Biochemicals. [G\textsuperscript{32}P]cAMP was obtained from New England Nuclear. All cyclic nucleotides and DEAE-cellulose (DE11) were from Sigma.

RESULTS

[3H]cAMP equilibrium binding to R\textsubscript{II} was attained in 45 min at 25°C in the absence or presence of certain competing cyclic nucleotides (Fig. 1). A virtually identical time for equilibrium was obtained for R\textsubscript{I} (not shown). Both isoforms bound nearly 2 mol of cAMP/mol of R subunit monomer as was recently demonstrated by several laboratories (2–4). The rate of [3H]cAMP dissociation from R\textsubscript{II} was determined by adding excess nonradioactive cAMP at the 45-min time point followed by assay of [3H]cAMP bound at various times thereafter (Fig. 2). Dissociation occurred in two phases for each isoform, indicating the presence of two distinct populations of binding sites. The site which slowly exchanged cAMP (Site 1) had a dissociation rate constant of 0.045 ± 0.002 (n = 6) min\textsuperscript{-1} for R\textsubscript{II} and 0.027 ± 0.002 (n = 4) min\textsuperscript{-1} for R\textsubscript{I}. The dissociation rate of the site with fast cAMP exchange (Site 2) was corrected for the contribution of Site 1 and had rate constants of 0.252 ± 0.010 (n = 5) and 0.237 ± 0.022 (n = 4) for R\textsubscript{II} and R\textsubscript{I}, respectively. It was also noted that the proportion of each component was 50 ± 5% in the experiments. In order to study cyclic nucleotide specificity, competing nonradioactive cyclic nucleo-
CAMP, on R3H]cAMP equilibrium binding to derivatives could also be demonstrated for left.

was to exaggerate the rapid component, indicating 8-Br-CAMP pattern was observed using 8-Br-CAMP except that the effect dissocating from Site 2. However, at 8-Br-cAMP showed a preference for Site 1, the effect being slightly greater for RI than for RII. It can also be seen that when the contribution of [3H]cAMP binding to Site 1 relative to the total [3H]cAMP binding to both sites became less (8-Br-cAMP curves), the measured rate of dissociation from Site 2 approached its true value as the curve shifted toward the left.

To further examine cyclic nucleotide specificity, different degrees of inhibition of [3H]cAMP binding to RII were achieved by varying the concentration of competing cIMP and 8-Br-cAMP (Fig. 3A). At a final concentration of 4.5 \( \mu \text{M} \) cIMP (23% inhibition), 40% of the total [3H]cAMP was still dissociating from Site 2. However, at 90 \( \mu \text{M} \) cIMP (75% inhibition), no detectable [3H]cAMP was bound to Site 2 as indicated by the absence of the rapid component. The same pattern was observed using 8-Br-cAMP except that the effect was to exaggerate the rapid component, indicating 8-Br-cAMP binding to Site 1. A similar family of curves using these derivatives could also be demonstrated for RII (not shown).

The effects of increasing concentrations of two other cyclic nucleotide analogues, 8-Br-cGMP and N\(_6\) O\(_2\)-dibutylry-cAMP, on [3H]cAMP equilibrium binding to RII are shown in

Fig. 3B. Inhibition of [3H]cAMP binding by 8-Br-cGMP and N\(_6\) O\(_2\)-dibutylry-cAMP was less than 50% at a 200-fold excess. Inhibition was only slightly greater than 50% when the excess was 2000-fold (not shown). This suggested a high degree of preference for Site 1 or Site 2, which is also shown in the dissociation curves in the inset (Fig. 3B).

The relative selectivity of various cyclic nucleotides for intrachain Sites 1 and 2 is shown in Table 1. cGMP inhibited [3H]cAMP binding to RII very poorly and did not alter the pattern of cAMP dissociation from RI. Cyclic UMP was a poor competitor for cAMP binding to both R subunits, but it showed a slight relative preference for Site 2. As discussed above, N\(_6\) O\(_2\)-dibutylry-cAMP behaved differently for the two isozymes, showing a marked relative selectivity for Site 2 of both RI and RII. 8-Br-cGMP and 8-azido-cAMP behaved qualitatively like 8-Br-cAMP. These nucleotides strongly selected Site 1 of both isozymes. When [3H]cAMP binding was inhibited greater
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TABLE 1
Relative selectivity of various cyclic nucleotides for intrachain Sites 1 and 2

<table>
<thead>
<tr>
<th>Competing cyclic nucleotide</th>
<th>Cyclic nucleotide concentration</th>
<th>Inhibition of [3H]cAMP binding</th>
<th>Expression of [3H]cAMP dissociation</th>
<th>Site selectivity compared with cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>%</td>
<td>R₁</td>
<td>R₁ (Site 1/Site 2)</td>
</tr>
<tr>
<td>H₂O</td>
<td>90</td>
<td>60</td>
<td>3</td>
<td>0.55</td>
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<tr>
<td>cGMP</td>
<td>800</td>
<td>75</td>
<td>66</td>
<td>0.50</td>
</tr>
<tr>
<td>cAMP</td>
<td>270</td>
<td>54</td>
<td>55</td>
<td>0.60</td>
</tr>
<tr>
<td>N₆, O₇-dibutyryl-cAMP</td>
<td>90</td>
<td>94</td>
<td>73</td>
<td>0.60</td>
</tr>
<tr>
<td>8-oxo-cAMP</td>
<td>9</td>
<td>82</td>
<td>78</td>
<td>0.50</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>18</td>
<td>87</td>
<td>59</td>
<td>None</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>90</td>
<td>40</td>
<td>28</td>
<td>None</td>
</tr>
<tr>
<td>8-azido-cAMP</td>
<td>270</td>
<td>40</td>
<td>28</td>
<td>None</td>
</tr>
<tr>
<td>cAMP</td>
<td>18</td>
<td>83</td>
<td>74</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>91</td>
<td>93</td>
<td>None</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of competing cyclic nucleotides in the [3H]-cAMP binding reaction on equilibrium binding activity and subsequent dissociation of [3H]cAMP from R₆. A, approximately 0.20 µM R₆ subunit was incubated (25°C) with 1 µM [3H]cAMP and other cyclic nucleotides included at the final concentrations shown. At equilibrium, cAMP inhibited [3H]cAMP binding by 23% (4.5 µM cAMP) and 75% (90 µM cAMP) and 8-bromo-cAMP inhibited 27% (0.46 µM 8-Br-cAMP) and 62% (9 µM 8-Br-cAMP). After the 40-min time point of the binding reaction, 5 µl of nonradioactive 10 µM cAMP was added and 40-µl samples were withdrawn and filtered at the times indicated. B, equilibrium [3H]cAMP binding was determined at 45 min (25°C) in reaction mixtures containing 50 µl of 1 µM R₆, 50 µl of 1 µM [3H]cAMP binding mix, and 10 µl of the nucleotides shown, which were added in concentrations which gave the final cyclic nucleotide concentration ratios indicated. Inset, experiments done exactly as described for A except that the final nucleotide ratio was 2006/8-Br-cAMP or N₆, O₇-dibutyryl-cAMP to 1 cAMP. The decrease in [3H]cAMP binding at these high nucleotide concentrations was 54% for 8-Br-cAMP and 62% for N₆, O₇-dibutyryl-cAMP. In B/Br = natural logarithm of [3H]cAMP bound + [3H]cAMP bound at the 45-min point (before the addition of nonradioactive cAMP).

subunit (2-4), little thought has been given to the possibility that each intrachain binding site may possess unique characteristics. Hoppe et al. (13) proposed a conformational change in isozyme I R upon cAMP binding and R-C dissociation which converted the binding site into one with greater affinity. The present studies demonstrate a clear difference in the characteristics of cAMP binding to each site on the same protein chain of purified R₁ and R₆. Although cAMP dissociation behavior of each isozyme was similar, some differences were observed between the isozymes in the ability of cyclic nucleotides to compete for cAMP binding and in their preference for one or the other of the two binding sites. Raddox et al. (14) noted that cGMP could more effectively compete with cAMP for binding to isozyme I protein kinase than to isozyme II. Activation of protein kinases I and II by various cAMP analogues was also different (15, 16). In spite of some differences in cAMP binding characteristics of R₁ and R₆, the similarities reported here were much more striking. This might suggest that even though the overall structures of the two R subunits are different in several respects, the two intrachain binding sites could have been conserved during evolution.

In our hands, cGMP was a poor inhibitor of cAMP binding to R₆ but competed well for cyclic nucleotide binding to both sites of R₁. However, the presence of a bromo group on position 8 of this nucleotide made it a much better competitor for binding to R₆ without changing it for R₁. However, binding of 8-Br-cAMP appeared to be selective for Site 1 of both R₁ and R₆. Nearly all 8-substituted nucleotides selected Site 1 of both isozymes. It is possible that Site 2 has less ability to accommodate such bulky groups in position 8. By the same reasoning, Site 1 could have less ability to accommodate derivatives with alteration at N₆.

It is also possible that a specific conformation of the nucleotide directs site selectivity. Bulky substitutions on position 8 are thought to result in a predominately "syn" configuration (13, 17). By contrast, N₆-substituted cAMP molecules are thought to be in an "anti" conformation (18). Thus, N₆, O₇-dibutyryl-cAMP may select Site 2 of R₆ for this reason. Perhaps one should consider the nature of the substitution, its position, and nucleotide conformation in site selectivity of each cyclic nucleotide. cIMP was a better competitor of cAMP binding to R₁ than to R₆, but this nucleotide strongly selected Site 2 of both isozymes.

subunit was incubated (25°C) with 1 µM [3H]cAMP, and 40 pl samples were withdrawn and filtered at the times indicated. A, equilibrium [3H]cAMP binding was determined at 45 min (25°C) in reaction mixtures containing 50 µl of 1 µM R₁, 50 µl of 1 µM [3H]cAMP binding mix, and 10 µl of the nucleotides shown, which were added in concentrations which gave the final cyclic nucleotide concentration ratios indicated. Inset, experiments done exactly as described for A except that the final nucleotide ratio was 2006/8-Br-cAMP or N₆, O₇-dibutyryl-cAMP to 1 cAMP. The decrease in [3H]cAMP binding at these high nucleotide concentrations was 54% for 8-Br-cAMP and 62% for N₆, O₇-dibutyryl-cAMP. In B/Br = natural logarithm of [3H]cAMP bound + [3H]cAMP bound at the 45-min point (before the addition of nonradioactive cAMP).
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The interpretations of the results reported here are subject to two important qualifications. First, even though cAMP dissociated from Sites 1 and 2 of both regulatory subunits at a much slower rate than that from Site 2, it is likely but not certain that cAMP has a higher affinity for Site 1 than for Site 2. Affinity is dictated by both association and dissociation rates. Secondly, since the selectivity of cyclic nucleotides for Sites 1 and 2 was determined in a competitive reaction, the selectivity was not absolute but relative to selectivity of cAMP. For example, even though cAMP had a relative selectivity for Site 2, this nucleotide might still possess an absolute selectivity for Site 1.

Dissociation of cAMP from one site could affect the rate of dissociation of the nucleotide from the other site within the same protein chain. However, this cooperative interaction as such is unlikely in face of the saturating cyclic nucleotide concentrations used in these experiments. Intrachain binding sites may strongly interact, however, since trace levels of [3H]cAMP (1 nM) incubated with cAMP-free R subunit (0.2 µM) is distributed in both sites in approximately equal amounts. Another possible explanation for this result is that cAMP may be bound in a dimeric form in a process involving a close approximation of Site 1 with Site 2. Under certain in vitro conditions, dimerization of cAMP molecules has been observed. Finally, an interconversion of one binding site to the other also cannot be ruled out.

Daskeland (20) observed two kinetically distinct populations of cAMP binding sites of unknown stoichiometry in partially purified isozyme I protein kinase or R subunit and proposed that each R subunit of a dimer is distinct with respect to cAMP binding. However, Chau et al. (21) recently found a single cAMP dissociation component in isozyme I R. Binding assay conditions could be important in studies of this type. We first reported (22) that the use of high ionic strength buffers and histone in the cAMP binding assay enhanced the amount and recovery of the R-3H]cAMP complex. Thus, it is now established that for pure R subunit each monomer contains two cAMP binding sites. The presence of two dissimilar intrachain binding sites in identical R monomers seems likely, particularly in view of the much higher probability of intrachain variability as compared with interchain variability of protein dimers. There is no existing evidence that the two chains of R dimer are not identical.

It is clear from these studies that any future investigations of protein kinase activation by cAMP or its analogues must consider the existence of two different intrachain binding sites per monomer of R. For example, the binding of 8-Ne-cAMP and cAMP-Sepharose to R may be site-specific. Possible interaction between intrachain as well as interchain sites and differences in their binding constants and specificities may be important characteristics in the intracellular action of cAMP.

REFERENCES


S. R. Rannels and J. D. Corbin, unpublished observations.

The use of site-selective analogues may enable one to determine which site, or sites, must bind cyclic nucleotide before activation of protein kinase occurs. Such information would be invaluable in understanding selective activation of protein kinase I or II in a particular tissue. It would also be important to determine whether or not in intact cells different cyclic nucleotides or different forms of cAMP bind to Sites 1 and 2.

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